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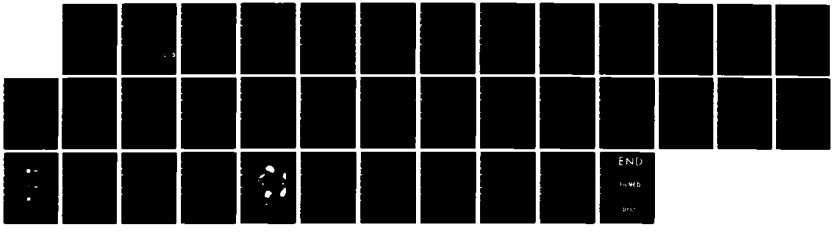

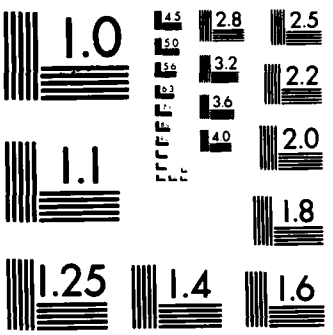

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DETECTION, ISOLATION AND CHARACTERIZATION OF AN AGENT FROM FEBRILE PATIENTS IN MALAYSIA SEROLOGICALLY REACTIVE WITH RICKETTSIA SENNETSU

FINAL COMPREHENSIVE REPORT

MIODRAG RISTIC

DECEMBER 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

DAMD17-81-C-1189

College of Veterinary Medicine
University of Illinois
Urbana, IL 61801

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June 25, 1985

Defense Technical Information
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Dear Sir:

Enclosed you will find the final comprehensive report of the U.S. Army Contract DAMD17-81-C-1189 entitled "Detection, Isolation and Characterization of an Agent from Febrile Patients in Malaysia Serologically Reactive with Rickettsia sennetsu."

I hope that you will find it satisfactory.

Sincerely yours,

Miodrag Ristic, DVM, PhD
Professor

MR/ml

Enclosures

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1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A157489
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) Detection, Isolation and Characterization of an Agent from Febrile Patients in Malaysia Serologically Reactive with Rickettsia sennetsu	5. TYPE OF REPORT & PERIOD COVERED Final Report 1 Oct. 1981-31 Dec.1983
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Miodrag Ristic	8. CONTRACT OR GRANT NUMBER(s) DAMD17-81-C-1189
9. PERFORMING ORGANIZATION NAME AND ADDRESS College of Veterinary Medicine University of Illinois Urbana, IL 61801	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102B510.AC070
11. CONTROLLING OFFICE NAME AND ADDRESS	12. REPORT DATE December 1983
	13. NUMBER OF PAGES 34
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release
Distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The possible role of Rickettsia sennetsu or R. sennetsu-like agents in the etiology of fever of unknown origin (FUO) among military and civilian populations in Southeast Asia has been examined. Seroepidemiologic studies on 1,208 serum samples obtained from patients with FUO residing in Malaysia and the Philippines revealed the presence of anti-R. sennetsu antibodies in 30% of the samples examined. Isolation studies using whole blood collected from some of the above seropositive patients were

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performed in an attempt to isolate the etiologic agent. A rickettsia-like agent (temporarily designated #11908) was successfully isolated from the blood of one of the above patients from Malaysia. Preliminary cross-serologic studies revealed the agent to be closely related, but not identical, to R. sennetsu

Biochemical and immunochemical characterization of purified R. sennetsu membrane proteins revealed the presence of two major and four minor proteins. Future studies are aimed towards the identification of the antigenic relationship between the Ehrlichia-sennetsu group agents.

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SUMMARY

This two year research contract has been carried out in support of research at the United States Army Medical Research Unit (USAMRU) in Kuala Lumpur, Malaysia. It concerns the role of Rickettsia sennetsu or R. sennetsu-like agents in the etiology of fever of unknown origin (FUO) among military and civilian populations in Southeast Asia. Seroepidemiologic studies on some 1,208 serum samples of patients with FUO residing in Malaysia and the Philippines revealed the presence of anti-R. sennetsu antibodies in 30% of the samples tested. More recently, a rickettsia temporarily designated #11908, was isolated from the blood of one of the above test patients from Malaysia. Preliminary cross-serologic studies of this agent with monospecific sera to major rickettsiae of human health importance, including R. sennetsu, failed to resolve the identity of this rickettsia organism. The agent, however, did cross react at relatively low titers with several of the rickettsiae, including R. sennetsu.

Major accomplishments described in this report concern: serologic investigations for R. sennetsu antibodies among populations of Malaysia and the Philippines; preliminary characterization of a rickettsia recently isolated from the blood of a serologically positive patient from Malaysia; biochemical and immunochemical definition of R. sennetsu and specific antigenic relationship to Ehrlichia canis of a protein fraction isolated from the R. sennetsu agent.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

ABSTRACT

The possible role of Rickettsia sennetsu or R. sennetsu-like agents in the etiology of fever of unknown origin (FUO) among military and civilian populations in Southeast Asia has been examined. Seroepidemiologic studies on 1,208 serum samples obtained from patients with FUO residing in Malaysia and the Philippines revealed the presence of anti-R. sennetsu antibodies in 30% of the samples examined. Isolation studies using whole blood collected from some of the above seropositive patients were performed in an attempt to isolate the etiologic agent. A rickettsia-like agent (temporarily designated #11908) was successfully isolated from the blood of one of the above patients from Malaysia. Preliminary cross-serologic studies revealed the agent to be closely related, but not identical, to R. sennetsu.

Biochemical and immunochemical characterization of purified R. sennetsu membrane proteins revealed the presence of two major and four minor proteins. Future studies are aimed towards the identification of the antigenic relationship between the Ehrlichia-Sennetsu group agents.

BODY OF THE REPORT

I. PROBLEM: Detection of Serum Antibodies to R. sennetsu in Human Populations in Malaysia and the Philippines

BACKGROUND

Rickettsia sennetsu is the causative agent of sennetsu rickettsiosis, a disease which was originally believed to be confined to Western Japan. Commonly observed disease symptoms include fever, malaise, headache, back pain, anorexia, lymphoadenopathy and insomnia (Misao and Kobayashi, 1954).

In 1954, Misao and Kobayashi made the first successful isolation of R. sennetsu by inoculating mice and human volunteers with the blood, bone marrow and lymph node aspirates from a patient during the acute phase of the disease. Since then, R. sennetsu has been successfully cultivated in vitro in Hela cells (Sugi, 1960), human anmiontic-membrane derived Fl cells (Minamishima, 1965), African green monkey kidney cells (Anderson et al., 1965), human blood monocytes (Hoilien et al., 1982), dog primary cell cultures (Holland et al., 1985), and most recently, in a continuous murine monocyte cell culture (P388D1) (Cole et al., 1985). The use of the above continuous murine monocyte cell culture has allowed for the production of R. sennetsu antigen on a large scale within a short period of time by utilizing the rapid synchronous generation time of the parasite and the host cell (Koren et al., 1975). This mass quantity of antigen not only allows for the initiation of various immunochemical studies but also provides sufficient material for use in the indirect fluorescent antibody test (IFA), which serves as a tool in epidemiologic studies of the disease caused by the agent. By examining human sera from various regions outside Japan, it may be possible to acquire greater knowledge of the agent and its geographic distribution, the disease which it causes and possibly, the means by which the agent is transmitted.

RESULTS

In this laboratory, 1,208 human serum samples from Malaysia and the Philippines have been examined for the presence of R. sennetsu antibodies using the IFA test. Of 634 serum samples received from Malaysia, 197 (31%) were positive for antibody to R. sennetsu. These sera were obtained from patients with symptoms of FUO. Similarly, 202 (35%) of 574 serum samples originating from various populations in the Philippines were also found positive for antibody to R. sennetsu (Table 1).

Table 1. Results of Indirect Fluorescent Antibody (IFA) Test for Rickettsia sennetsu and Ehrlichia canis with Human Sera from Southeast Asia.

Group	Country of Origin	Number of Cases	Number of Positive	Percentage Positive
1	Malaysia	200	58	29.0
2	Malaysia	88	23	26.1
3	Malaysia	40	11	27.5
4	Philippines (Sulawesi)	196	86	43.9
	Philippines (Chinese Marines)	188	38	20.0
	Philippines (Mosbate)	200	68	34.5
5	Malaysia	50	17	34.0
6	Malaysia	204	73	35.8
6b	Malaysia	204	39*	-
6c	Malaysia	204	86**	42.1

*Suspect positive at 1:10 dilution
**Positive for Ehrlichia canis

CONCLUSIONS AND RECOMMENDATIONS

The verification and distribution of R. sennetsu beyond Western Japan has not previously been documented. The above serological findings show that there is a high incidence of sennetsu rickettsiosis or a disease caused by a similar agent in Malaysia and the Philippines (Table 1). The IFA antibody titers range from 1:20 to 1:640 with the majority of seropositive samples reacting at titers within the 1:10 to 1:40 dilution range (Table 2).

From the serological data, one may conclude that sennetsu rickettsiosis is not limited to Japan alone, but is present in other regions of Southeast Asia. A more comprehensive definition of the various factors associated with the disease, along with determining the means by which the disease is transmitted, would serve to increase our knowledge with regard to this disease and thereby develop preventative measures.

Table 2. Percentage of Positive Indirect Fluorescent Antibody Tests for *Rickettsia sennetsu* with Human Sera from Southeast Asia.

Group	IFA Result	Positive Tested	Percentage titer of Positive Samples 1:10	1:20	1:40	1:80	1:160	1:320	1:640
1	29%	58/200	7	13.5	5	3	0.5	-	-
2	26.1	23/88	1	2.3	5.7	6.8	7.9	-	2.3
3	27.5	11/40	15	7.5	2.5	2.5	-	-	-
4a	43.9	86/196	21.4	16.3	6.1	-	-	-	-
4b	20.0	38/188	7.9	9.6	1.6	0.5	-	-	-
4c	34.0	68/200	21.5	8.5	1.0	1.5	1.0	0.5	-
5	34.0	17/50	14.0	8.0	4.0	-	4.0	2.0	2.0
6	35.8	73/204	5.4	16.7	11.3	1.9	0.5	-	-

II. PROBLEM: Isolation of a Rickettsia from Whole Blood of a Patient Serologically Positive to R. sennetsu with Symptoms of FUO

BACKGROUND

Misao and Kobayashi (1954) were the first to isolate R. sennetsu by inoculating mice with the blood and bone marrow homogenates of a patient suffering from "Japanese infectious mononucleosis." Tanaka and Hanaoka (1961) later described ultrastructural and taxonomic properties of R. sennetsu. In their study, they maintained and propagated the organism in dd/s mice and found that immature peritoneal macrophages were more susceptible to infection by the rickettsiae than were mature macrophages.

In a study reported by Tachibana et al (1979), R. sennetsu has been detected in the cytoplasm of endothelial cells lining splenic blood capillaries at 3 days following intraperitoneal inoculation of the agent and, after 7 days, within the resident macrohages of the spleen. Bourgeois et al (1977), in their study on the epidemiology and serology of scrub typhus among Chinese soldiers, were able to isolate R. tsutsugamushi from peritoneal macrophages of several groups of mice following inoculation of whole blood taken from patients suffering from an acute form of scrub typhus.

Based upon the above findings on the isolation of R. sennetsu and R. tsutsugamushi and, in view of the fact that the development of a serologic test has allowed for the detection of antibody to R. sennetsu, it has been deemed necessary to attempt isolation of the above or a related agent from seropositive patients suffering from FUO.

APPROACH

1. Inoculation of blood samples: Eight whole blood samples randomly selected from a total of 51 such samples received from Col. George E. Lewis, Jr. (Commander, U.S. Army Medical Research Unit, Kuala Lumpur, Malaysia) were individually mixed with 0.50 ml of veronal buffer saline - phosphate glutamate buffer, pH 7.4. Each sample was inoculated into groups of 4 mice. Seven to 9 days post inoculation, the mice were sacrificed by cervical dislocation and 3-4 ml of Hanks Balanced Salt Solution (HBSS) containing 10 ul/ml of heparin was injected into the peritoneal cavity of each mouse. Abdomens of the

mice were massaged for 2-3 minutes prior to aseptic removal of the peritoneal lavage using a syringe equipped with a 25 guage needle. The aseptically collected peritoneal fluid was then centrifuged at 600 x g for 10 minutes and the supernatant fluid discarded. The pellet was resuspended with culture media consisting of Eagle's Minimum Essential Medium (MEM) with Earle's salts, 1% L-glutamine, 4% sodium bicarbonate (7.5%) and 10% heat inactivated fetal bovine serum (FBS). One-half of the cell suspension was planted in Leighton tubes containing coverslips, while the other half was used to inoculate semiconfluent P388D1 murine macrophage cell cultures in triplicate sets.

2. Cell cultures: The P388D1 strain of a murine-monocyte macrophage cell line was obtained from ATCC and maintained by continuous passages in this laboratory. Cell culture media consisted of Eagle's Minimum Essential Medium (MEM) with Earle's Salt, supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% L-glutamine. Cell monolayers were propagated in 25 cm^2 tissue culture flasks. By this procedure, cell monolayer confluency is reached by the third to fourth day of incubation at 37° C in air with 5% CO_2.

3. Detection and isolation of a Rickettsia-like agent: Media from the semiconfluent cell monolayers of P388D1 were removed and each culture was inoculated with 1 ml of the resuspended harvested peritoneal macrophages. The inoculated cultures were incubated at 37° C for 4-6 hours to allow for adherence of the peritoneal macrophages onto the monolayer. At this time, 4 ml of fresh culture media was added to each flask. Cultures were then placed in a 37° C incubator equipped with 5% CO_2 in air. Approximately 48 to 60 hours later, when cell monolayers became confluent, cultures were washed twice with 2 ml of 2.5% trypsin-EDTA. Cultures containing 1.5 ml of Trypsin-EDTA were then incubated at 37° C for 5 minutes at which time the monolayer was dislodged from the flask by gentle agitation, and then split 1:3. After 3-4 days, cultures were examined for infection and confluency before being split again as previously described.

4. Examination of inoculated cell cultures for the presence of an agent: One ml of supernatant fluid from inoculated cultures was placed into a round 5 ml flat bottom vial containing a coverslip 12mm in diameter. The

vial was centrifuged at 700 x g for 15 minutes and the supernatant fluid was discarded. The coverslip was then removed, stained by the Giemsa method, air dried, mounted cell side down onto a microscope slide using permount and examined microscopically for the presence of infection.

RESULTS

Of the 8 blood samples studied, rickettsia-like organisms were detected in one sample (ID#11908) beginning at the 4th consecutive passage of the inoculated P388D1 cell cultures. At this time, the above cell cultures appeared to contain a very scarcely distributed rickettsia-like agent within the cell cytoplasm. Other samples cultured and treated in the same manner showed no indication of infection. All cultures were subjected to six additional passages. Prior to each passage, all cultures were examined microscopically. At the 5th, 6th and 7th passages, sample #11908 showed consistent quantities of rickettsia-like organisms located within cytoplasmic vacuoles of the cultured cells. An interesting finding in the above cultures was an enhancement of vacuole formation in comparison to cell cutures lacking any evidence of infection. An examination of the 9th consecutive passage of sample #11908 provided conclusive evidence of infection in that an abundant growth of a rickettsia-like agent was observed. The organism was found to occur either singly or in nest-like clusters in vacuoles throughout the cytoplasm of the cultured monocytes. Other culture samples were maintained through 14 passages with no evidence of infection before they were discarded.

The serological findings on the isolated agent #11908 by means of IFA are shown in tables 3 and 4. In this study, specific rabbit-sera against rickettsiae of major human importance were examined for cross-reactivity with the #11908 agent*. The study revealed cross-reaction of the newly isolated agent with R. tsutsugamushi (Gilliam) strain at the titer of 1:80, the Karp strain reacted at a titer of 1:40 and there was no cross reaction with the Kato strain. The agent also cross reacted with Rochalimaea quintana and R.vinsonii at

*Sera kindly provided by Dr. Emilio Weiss of the Naval Research Insitute of Bethesda, MD.

Table 3. Serologic Cross Reactions Between Rabbit Anti-sera to Various Rickettsiae of Human Importance and Rickettsia #11908 Antigen Using the Indirect Flourescent Antibody (IFA) Test.

Rabbit Anti-sera	IFA Titer for Rickettsia #11908
Rickettsia prowazeki	Negative
Rickettsia tsutsugamushi (Gilliam)	1:80
Rickettsia tsutsugamushi (Karp)	1:40
Rickettsia tsutsugamushi (Kato)	Negative
Rickettsia canada	1:20
Rickettsia conorii	1:80
Rickettsia akari	1:20
Rickettsia typhi	Negative
Rochalimaea quintana	1:80
Rockalimaea vinsonii	1:40

Table 4. Serologic Cross-Reaction Between Anti-sera to *Rickettsia sennetsu* and *Ehrlichia canis* Against *Rickettsia* #11908 Using the Indirect Fluorescent Antibody (IFA) Test.

Agent	Source of Anti-sera	IFA Titer					
		Homologous	Rickettsia #11908				
			1:10	1:20	1:40	1:80	1:160
R. sennetsu	Rabbit	1:1280	+	+	+	±	±
E. canis	Dog	1:5120	+	+	+	±	±

+ = positive; ± = partially positive

titers of 1:80 and 1:40, respectively. These and other cross-reacting titers are shown in table 3. Table 4 shows the serologic cross-reaction between R. sennetsu and E. canis antibodies and the newly isolated rickettsia agent. Both organisms showed significant cross-reactivity with the agent #11908 at a titer of 1:40.

CONCLUSIONS AND RECOMMENDATIONS

The use of the macrophage-like cell culture line P388D1 has proven to be a suitable and convenient method for the isolation of various intracellular organisms. Baca et al (1981) were the first to use murine monocyte line P388D1 for successful propagation of Coxiella burnetii. This culture line has been successfully adapted in our laboratory for the continuous propagation of R. sennetsu (Cole et al., 1985). The relatively short generation time (20 hours) of these monocytes, along with their enhanced phagocytic properties (Koren et al., 1975) provides optimal conditions necessary for rapid intake and multiplication of the above agent.

Examination of the patient's record shows that sample #11908 originated from a 35-year-old Malaysian male settler in Jengka 18. The patient was admitted to the Montekah Hospital on the 10th day of his illness with a fever of 104° F, rigor, chills, headache, retrobulbae, muscle and back pain and abdominal cramps. Clinical signs consisted of rash, pharyngitis, lymphadenopathy and dehydration. Patient's blood was collected for serological and cultural studies. Serological evaluations of the patient revealed him to be negative for R. tsutsugamushi, R. prowazeki and R. rickettsii, but positive at titers of 1:20 for both R. sennetsu and E. canis using the respective IFA tests. The patient was also negative for leptospirosis, maleodosis, WF-OXK (Weil-Felix) and Widal, "O."

Based upon preliminary serological studies with several known rickettsiae, the agent in question, #11908, does not appear to be a prototype of R. sennetsu. Consequently, additional immunological and other studies to include ultrastructural and biochemical characterization will be needed before the true identity of the agent may be revealed.

In our previous report it was stated that Ehrlichia Sennetsu-like agents may contribute to the pathogenesis of yet poorly understood human disease syndromes. While serologically cross-reacting with several rickettsiae, the newly isolated agent appears similar to R. sennetsu on the basis of its growth pattern in cell cultures. Consequently, it is possible that this new agent represents another addition to the Ehrlichia-Sennetsu group of agents of potential importance to human health. More specific information on this subject should become available through studies proposed in this research contract request. In the meantime, further joint efforts on isolation and identification of additional potential etiologic agents of FUO must continue.

III. PROBLEM: Biochemical and Immunological Analysis of R. sennetsu Antigen and Specific Antigenic Relationship to E. canis of a Protein Fraction from R. sennetsu

BACKGROUND

Cell mediated immunity plays an important role in the defense against typhus and other rickettsial agents (Jerrells et al., 1975; Murphy et al., 1979; Shirai et al., 1976). Studies with experimentally infected animals indicate that immunity to R. typhi and R. tsutsugamushi is cell mediated and possibly independent of the humoral antibody response (Murphy et al., 1979; Shirai et al., 1976). Gambrill and Wisseman (1973) in their studies on the mechanism of immunity in typhus concluded that the destruction of typhus rickettsiae is dependent upon sequential interaction of the rickettsiae with both the humoral and the cellular factors. Lewis (1977), Lewis and Ristic (1978 a,b) and Kakoma (1980) also concluded that the maximal destruction of E. canis depends on the interaction between humoral and cellular immune responses.

Rickettsiae are obligate intracellular parasites and the basic structural features as seen by electron microscopy generally resemble those of gram negative bacteria with a demonstrable outer cell wall, periplasmic space, and inner trilaminar cytoplasmic membrane (Anacker et al., 1967; Brinton et al., 1971; Smith and Winkler, 1979). Purified outer membrane layers of R. prowazakii are rich in 2-keto-3-deoxyoctulosonic acid, a compound apparently unique to gram negative bacteria.

Silverman et al (1978) demonstrated by electron microscopy a microcapsule slime layer that is largely polysaccharide in nature in R. prowazekii and R. rickettsii, and postulated that this may be the focus of some major group-specific antigens which may function as an anti-phagocytic factor or aid in the attachment of rickettsiae to the potential host cells or both.

Rickettsia sennetsu morphologically differs from classic rickettsiae in that it does not occur freely in host cell cytoplasm but rather singly or in multiple forms within cytoplasmic membrane-lined vacuoles (Anderson et al., 1965;

Shirakoma, 1967). This characteristic has been observed in Coxiella burnetii (Handley et al., 1967) and in E. canis (Hildebrandt et al., 1973).

Ristic et al (1981) demonstrated a serologic relationship between R. sennetsu and E. canis. Since sennetsu rickettsiosis is an important human disease with possible occurrence beyond Western Japan, and since there is limited information regarding the mechanism of protective immunity, it was deemed necessary to study the biochemical and immunological properties of this agent and its relationship to E. canis. Moreover, such studies were deemed important as baseline information toward identification and characterization of a recently isolated R. sennetsu-like agent and other potential agents that may yet be isolated from patients with FUO.

APPROACH

1. Production of R. sennetsu: The Miyayama strain of R. sennetsu, which was originally isolated from a patient by Misao and Kobayashi (1954), was obtained from the American Type Culture Collection. This organism was propagated continuously in the murine macrophage cell line P388D1. The culture medium consisted of Eagle's Minimum Essential Medium (MEM) with Earle's Salt, supplemented with 10% heat inactivated fetal bovine serum (FBS), 4% sodium bicarbonate and 1% L-glutamine. Cultures were maintained at 37° C in air with 5% CO_2. Other aspects of cultivation of the organism and the method used for mass generation of its antigen have been described earlier in this report.

2. Collection and purification of R. sennetsu antigen: Supernatant medium was first removed from highly infected R. sennetsu P388D1 cell cultures. Monolayers were then harvested using trypsin-versene and together with the supernatant medium were centrifuged at 7,500 x g for 20 minutes. The pellet was washed twice with K36 (Weiss, 1967) buffer pH 7.2 and resuspended in 10 ml of the same buffer. The infected cells were then homogenized using a Dounce tissue homogenizer. The homogenate was subjected to three differential centrifugations in order to separate and collect free rickettsiae. The final supernatant which still contained a few host cells was later purified by the linear renograffin density gradient procedure of Weiss et al. (1975). Fractions of 1.2 ml were collected from the top of the tubes and an OD reading at 420 nm was made for each

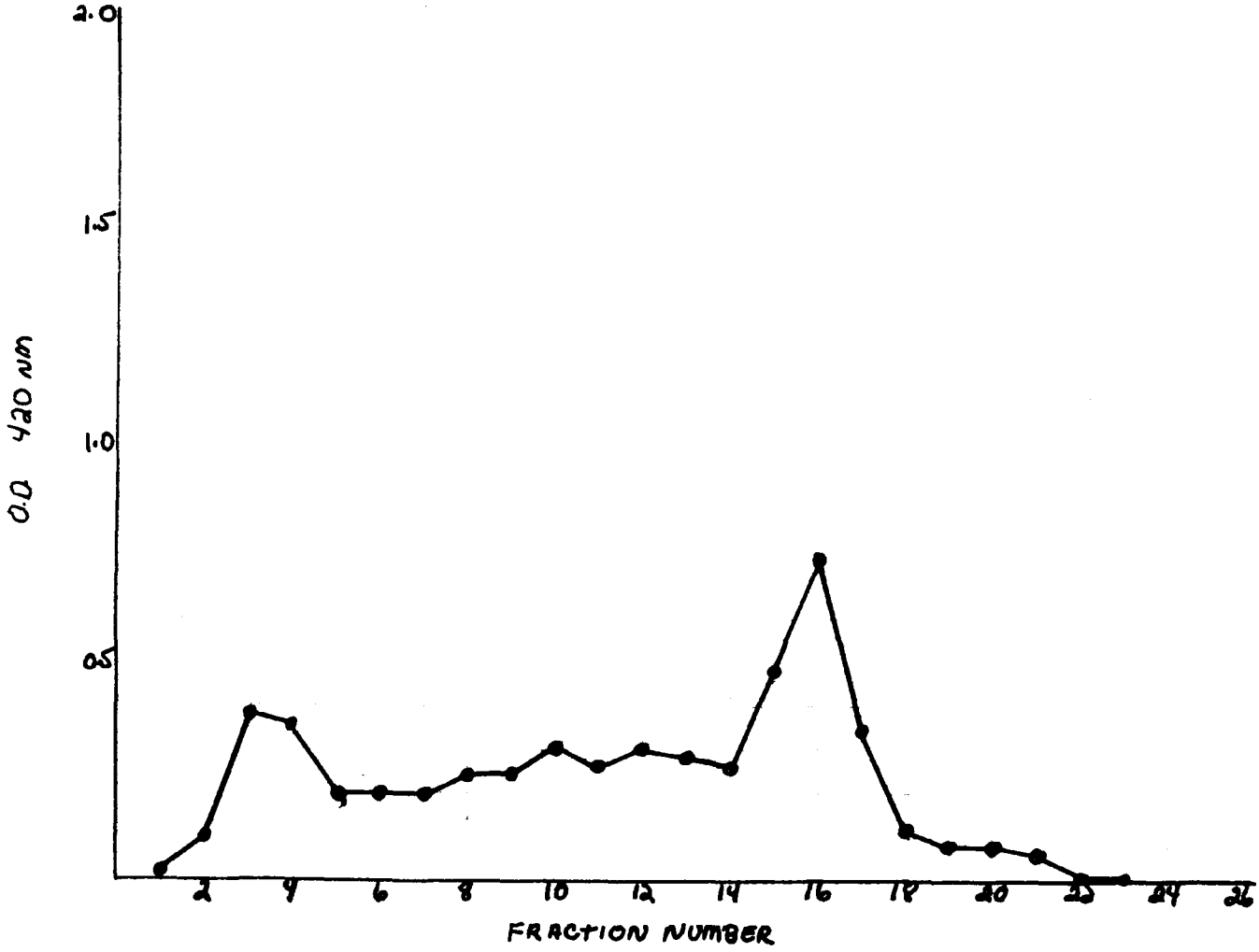

Figure 1. Profile of Purified Rickettsia sennetsu in Linear Renograffin (20-45% wt/vol in K36 buffer) Gradient.

fraction. Fractions containing the rickettsiae (fractions #2-5 and #14-19; Fig. 1) were pooled separately and pelleted by centrifugation at 20,000 x g for 30 minutes in a Sorvall S34 rotor. The pellet was washed 4 times to remove the renograffin and then resuspended in K36 buffer. The presence of purified rickettsiae in each fraction was confirmed by the IFA test.

3. Preparation of rabbit anti-R. sennetsu serum: Half of the volume of the renograffin-purified R. sennetsu was used to prepare rabbit anti-R. sennetsu antibody. Approximately 15 ug of the purified antigen was injected intravenously via rabbit ear vein once a week for 4 weeks. The rabbits were bled just prior to the final immunization. Ten days after the last immunization, a booster injection of 50 ug antigen mixed with Freund's complete adjuvant was given subcutaneously. Ten to twelve days later the rabbits were bled for the collection of sera.

4. SDS-Polyacrylamide gels: Rickettsia sennetsu proteins were electrophoresed on 10% acrylamide gel in a continuous phosphate buffer system described by Weber and Osborn (1969).

5. Crossed Immunoelectrophoresis: Crossed immunoelectrophoresis (CIE) was performed using the method of Weeke (1973). Samples containing 25 ug of protein were electrophoresed in the first dimension on 9.3 by 8.3 cm slides covered with 9 ml of 1% agarose in barbital buffer (pH 8.6 ionic strength of 0.05) and 0.02% sodium azide at 10V/cm for 75 minutes at 8° C in an LKB miltiphor cell (LKB Instruments, Inc., Rockville, MD). Electrophoresis was conducted overnight in the second dimension at 2V against 0.5 ml of hyperimmune rabbit anti-R. sennetsu and dog anti-E. canis sera in 10 ml of 1% agarose. The slides were washed in two changes of saline (0.85% NaCl) and two changes of distilled water, dried and then stained for 20 minutes with 0.5% Coomassie blue R-250 (BioRad Laboratories, Richmond, CA) in ethanol-acetic acid-water (9:1:9) and destained with 2% acetic acid.

6. Extraction of R. sennetsu membrane protein: Purified R. sennetsu (15 mg of protein) were suspended in 5 ml of 0.01M sodium phosphate pH 8.0 containing 0.15 M NaCl (PBS). This suspension was divided into 2.5 ml portions and centrifuged at 17,300 x g for 30 minutes at 4° C. One pellet was then

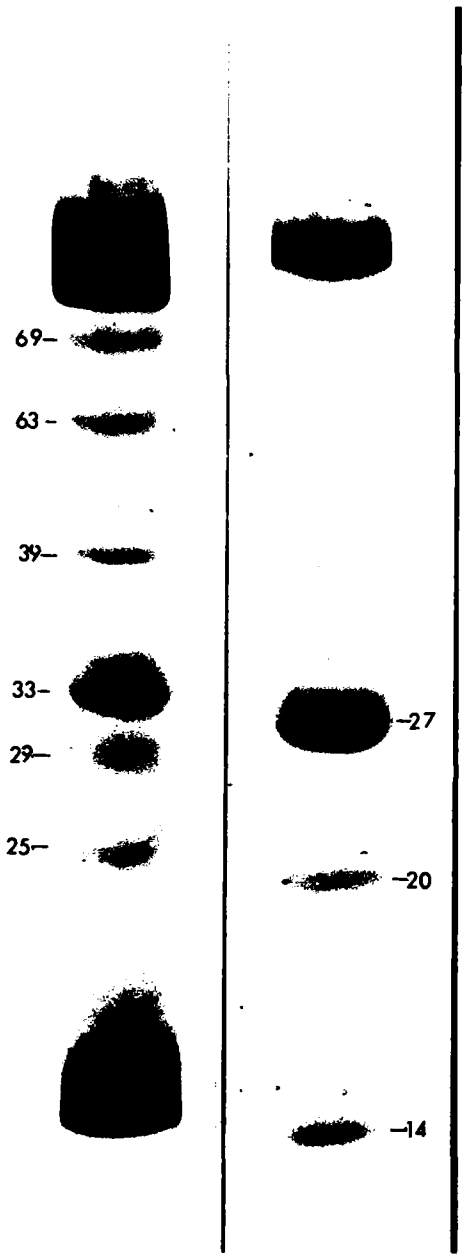

Figure 2. Protein analysis of Ricksttsia sennetsu antigen by means of SDS-PAGE

resuspended with 2.5 ml of PBS containing 1.5mM EDTA and 2% SDS and the other was resuspended in 2.5 ml PBS and 2% Triton X-100 (Sigma) in place of the SDS. The suspensions were sonically disrupted before being incubated at 37° C for 1 hour with occasional agitation. The soluble supernatants were collected after centrifugation and the pellet once more re-extracted using the same detergents. The supernatants were pooled, concentrated through an Amicon (Lexington, MA) ultrafiltration system using a 10,000 MW membrane. The insoluble pellets were each resuspended in 2 ml of the buffer and then exposed to sonic oscillation at 37.5W with pulse at 40% output for 10 minutes. The sonicate was centrifugated at 7,700 x g for 15 minutes and the supernatant was collected, aliquoted in 0.2 ml amounts and then stored at -80° C.

RESULTS

The gradient profile of purified R. sennetsu is shown in figure 1. Both peaks were free of host materials as verified by the IFA test. The dimension of fractions #14-19 compared to fraction #2-5 are slightly greater.

Molecular weights were determined for each polypeptide band revealed in the PAGE system using BioRad (Richmond, CA) low molecular weight standard. There were three major bands obtained from the triton X-100 extracted antigen with approximate molecular weights of 27,500, 20,000 and 14,000, respectively. The SDS extracted antigen had six bands out of which there were 2 major bands and 4 minor bands. The major bands revealed molecular weights of 63,000 and 33,000, while the minor bands exhibited molecular weights of 69,000, 39,800, 29,500 and 25,000, respectively (Fig. 2). In the sonicated antigen, two high intensity protein bands with molecular weights of 61,600 and 29,000 were observed, in addition to three lower density bands of 57,500, 46,000 and 37,000 MW.

Preliminary results with the CIE shows a consistent precipitin line with the sonicated samples when rabbit anti-R. sennetsu antibody was added to the agarose in the second dimension. When this was reacted with dog anti-E. canis antibodies there were two precipitin lines with the smaller line being identical to one of those found with the rabbit anti-R. sennetsu antibody.

CONCLUSIONS AND RECOMMENDATIONS

Purification of rickettsiae and other intracellular microorganisms, such as chlamydia, by the renograffin method has proven to be more efficient with less contamination of host material than purification using the sucrose gradient system (Caldwell et al., 1981; Dasch et al., 1981). The observed bands, i.e., fractions #2-5 and fractions #14-19 may represent two different populations of R. sennetsu considering the pleomorphic characteristics of this organism (Anderson et al., 1965), or it might be that R. sennetsu exhibited the same phenomenon in which the heavy band fractions #14-19 represent partly degenerated fragments of the light band.

Treatment of the purified R. sennetsu antigen with 2% triton X-100 in PBS with 1-5mM EDTA enabled the dissociation of some of the major outer membrane proteins while treatment with SDS which is used to solubilize inner membrane proteins (Tamura et al., 1974) resulted in the solubilization of six of these membrane proteins. One of the inner membrane protein, having a MW of 29,000, appears to be similar to one of the outer membrane proteins, whereas the remaining two outer membrane proteins seem to be unique. Comparison of the SDS treated antigen with the sonicated antigen shows one of the major proteins in the SDS, approximately 63,000 MW to be similar to one of the major proteins in the sonicated antigen, which approximated 62,000 MW.

Preliminary experiments using dog anti-E. canis antibody in the second dimension of the CIE against the extracted R. sennetsu antigen indicates cross-reactivity between the two organisms. Experiments directed towards identification of the antigenic relationship between R. sennetsu and E. canis utilizing the CIE with an intermediate E. canis antibody sandwiched between R. sennetsu antibody and antigen is currently being carried out. Further biomolecular characterization of the relationship between Ehrlichia-Sennetsu group agents should assure an aquisition of better understanding of the nature of this group of agents.

IV. APPENDIX

While this report was being written, the second isolation of a rickettsia was made from another patient with fever of unknown origin (FUO) in Malaysia. This time there was no need for a series of blind culture passages to be made before the presence of the organism became apparent. In fact, this second isolate was already present in the cytoplasm of peritoneal macrophages of a group of mice inoculated with a whole blood sample of the above patient. Many of the organismal forms (see figure 3 attached) strongly resemble immature and mature E. canis and/or R. sennetsu inclusion bodies. The patient from whose blood this organism was isolated is identified as a Malaysian male, 47 years of age, with symptoms of headache and rash. His occupation at the time was recorded as working at odd jobs. Paired sera (#12283 and 12310), collected within a four day period, each revealed antibody titers of 1:40 to R. sennetsu antigen using the IFA test.

This second isolation of a rickettsia from the blood of a patient serologically positive to R. sennetsu and negative to other common disease agents strongly substantiates the original concept that R. sennetsu or similar agents might be responsible for the occurrence of a disease syndrome currently referred to as FUO. As in the case of the first patient, clinical symptoms of this second individual are very typical of those described as sennetsu fever in Japan. Accordingly, it is indicated that this or a similar disease may not be limited to Japan but occurs in other regions of Southeast Asia, i.e., Malaysia. Further studies aimed at characterization of these two isolants and their pathogenic potential should contribute to a better understanding of the disease caused by Ehrlichia-Sennetsu group of agents.

Figure 3. Newly isolated Rickettsia from the blood of a patient with fever of unknown origin (Fig. 3A & 3B). Note the organisms in the cytoplasm of peritoneal macrophages of a mouse inoculated with the blood of the above patient (Fig. 3A). Inclusion bodies similar to those of R. sennetsu and/or E. canis are typical of this isolant (Fig. 3B-arrow).

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